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Targeted Therapies in Melanoma: Knowledge, Resistance and Perspectives

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Abstract

Several molecular mechanisms appear to play a major role in melanoma genesis and progression. Current targeted therapies focus on contrasting the activation of RAS/RAF/MEK/ERK and, to a less extent, PI3K/AKT pathways. Development of inhibitors of key effectors (mainly, BRAF mutant and MEK) has significantly improved treatment of patients with advanced melanoma. However, only rarely tumours present a durable regression due to a large variety of acquired and intrinsic mechanisms that drive resistance to the main targeted inhibitors. All these evidence suggest that in melanoma, as probably in all types of cancer, use of a combinatorial treatment approach, instead of targeting a single component of melanomagenesis pathways, could delay or prevent the emergence of resistance mechanisms responsible of tumour relapse. In this sense, a crucial step is thus represented by the full knowledge of such molecular mechanisms.

Keywords: Melanoma; MAPK pathway; Targeted therapy; Drug resistance

Introduction

Molecular mechanisms underlying pathogenesis of melanoma are complex. Single genetic or epigenetic alterations are not crucial person; rather, the interaction of some or most of such modifications may participate into the development and progression of the disease as well as contribute in generating distinct biological subsets of melanomas with different clinicopathological behaviors. Specific alterations have been described as deeply involved in melanomagenesis: induction of cell proliferation and/or impairment of the mechanisms controlling the melanocyte senescence (both promoting primary clonal selection and expansion), and suppression of the apoptosis (sustaining the cancer cell survival and tumor progression).

According to such a complex scenario, targeting a single component of the multiple pathways involved in pathogenesis is unlikely to yield a durable anti-tumor response in melanoma patients. Indeed, activation of alternative pathogenetic effectors is at basis of the development of resistance to target inhibitors.

Among others, the cascade of Ras, Raf, Mek and Erk proteins - which constitutes the mitogen-activated protein kinase (MAPK) pathway-has been reported to play a crucial role in melanoma pathogenesis [1]. Indeed, the ERK1/2 proteins have been found to be constitutively activated in melanoma, mostly as a consequence of mutations in upstream components of the pathway and their increased activity has been implicated in rapid cell growth as well as enhanced cell survival and resistance to apoptosis [1]. On this regard, activating mutations in BRAF and NRAS genes were found in approximately 45% and 15% of all melanomas, respectively (somatic mutations in such genes are mutually exclusive [2,3].

Treatment of patients with advanced melanoma has actually several effective options. Targeted therapy with BRAF inhibitors (vemurafenib, dabrafenib) or MEK inhibitors (trametinib) as well as immunomudulatory compounds [the anti-CTLA4 agent (ipilimumab) and the anti-PD-1 or anti-PD-L1 agents (nivolumab, lambrolizumab,MPDL3280A)] are all associated with improved clinical benefits, thus allowing to overcome the ineffectiveness of the conventional therapies [4].

Vemurafenib and dabrafenib have shown to benefit patients with BRAF activating mutation through achievement of a rapid tumour shrinkage in the majority of cases [5]. Treatments with both these drugs improve response rates and progression-free survival (PFS), with a favourable impact on overall survival (OS) [5]. MEK inhibitors alone or combined with a BRAF inhibitors have been recently demonstrated to exert a similar clinical efficacy [6].

Vast majority (up to 80%) of melanoma patients carrying BRAF mutations shows clinical and pathological response to therapy-with different rates of tumour reduction-when treated with either a BRAF inhibitor or a MEK inhibitor [7,8]. However, most of them develop resistance within 6-8 months after treatment initiation, as consequence of reactivation of the MAPK pathway or activation of alternative signalling pathways [9-13]. Nevertheless, a fraction of cases are primarily refractory due to an intrinsic resistance to such inhibitors [13].

The development of tumor resistance to single targeted agents appears inevitable and, given the high clinical responses, it is of pivotal importance to identify alternative therapies that overcome this problem [9-13].

Targeted Therapies and MAPK Pathway Components

Although majority of molecular mechanisms involved into the development, progression, and resistance-to-therapy of melanoma remains still largely unknown, several genes and cell-signalling

pathways have been implicated [14]. Canonical activation of MAPK pathway occurs when stimulation of the growth factor receptor leads to the activation of RAS family member (H-, N- or KRAS). Activated RAS interact with RAF isoform (A-, B- or CRAF) with consequent activations of RAF notably, RAF activation appears only after the formation of homo–or heterodimers between different isoform, that lead to the phosphorylation of MEK which activates ERK through a phosphorylation event [14-19].

Despite RAS has been largely implicated in tumour initiation and promotion, RAS itself has not become a successful target of therapy [20,21]. The strategies used to develop drugs able to inhibit the RAS activity are aimed at preventing its interaction with several components of the upstream or downstream signalling pathways regulated by this protein [21]. In this sense, a promising way of interfering with Ras function seemed to be the inhibition of farnesyltransferase, the enzyme coupling a 15-carbon isoprenyl group to Ras proteins, by farnesyltransferase inhibitors. The block of farnesylation markedly impairs the functioning of active RAS protein [22]. While a good in vitro antitumour activity has been reported in human melanoma cell lines, with downregulation of ERK and/or AKT and induction of apoptosis [22,23], farnesyltransferase inhibitors have always failed to be effective in melanoma patients (even if all cohorts treated with these agents were never selected for the activated-RAS status [24,25]). Lonafarnib, a recently discovered farnesyltransferase inhibitor, did not significantly inhibit growth of metastatic melanoma cells nor sensitize melanoma cells to the chemotherapeutic agents tested. In contrast, lonafarnib significantly augmented the growth inhibitory effects of the pan-RAF inhibitor sorafenib, inducing marked apoptosis and abrogated invasive melanoma growth [26]. Therefore, combination of farnesyltransferase inhibitors with other pathway-targeted drugs or, alternatively, a more stringent selection of the patients' cohorts could be helpful to increase the clinical efficacy of such compounds.

Therapeutic strategies have thus been focused on inhibiting downstream effectors of the RAS-driven pathways, MAPK and PI3K-AKT. Table 1 summarizes the main targeted agents introduced in clinical practice, as registered into the ClinicalTrial.gov database of the U.S. National Institutes of Health (at the https://clinicaltrials.gov/).

Target	Clinical agents	Activity
BRAF	Vemurafenib Dabrafenib LGX818 RO5212054	Selectively binds to and inhibits activated BRAF, inhibiting the proliferation of tumor cells with mutated BRAF gene
MEK	Trametinib Selumetinib Pimasertib TAK-733MSC2015103B	Binds to and inhibits MEK 1 and MEK 2, resulting in inhibition of growth factor-mediated cell signaling and tumor cell proliferation
	Cobimetinib	Binds to and inhibits the catalytic activity of MEK1, resulting in inhibition of activating ERK2 phosphorylation and tumor cell proliferation
	RO4987655	Binds to and inhibits MEK 1, which may result in inhibition of MEK-dependent cell signaling and tumor cell proliferation
Dual MEK-RAF	RO5126766	Specifically inhibits kinase activities of Raf and MEK, resulting in inhibition of target gene transcription that promotes malignant cell transformation
Pan-RAF	Sorafenib	Blocks RAF kinase (regardless of mutation status) and other kinases that control cell division and proliferation
	RAF265	Binds and inhibits Raf kinases and VEGFR-2, which may result in reduction of tumor cell growth and proliferation
PI3K	BKM120 XL147 ZSTK474 PX-866 GDC-0941	Reversibly binds to class 1 PI3Ks in an ATP-competitive manner, inhibiting the production of PIP3 and activation of the PI3K signaling pathway; this may result in inhibition of tumor cell growth and survival in susceptible tumor cell populations
AKT	MK2206 GSK2110183 GDC-0068	Binds to and inhibits AKT in a non-ATP-competitive manner, resulting in inhibition of the PI3K/AKT signaling pathway and tumor cell proliferation and induction of tumor cell apoptosis
mTOR	AZD8055 TemsirolimusRidaforolimus	Binds to and inhibits mTOR, resulting in decreased expression of mRNAs necessary for cell cycle progression and arresting cells in the G1 phase of the cell cycle
	Sirolimus	Binds to FKBP-12 to generate an immunosuppressive complex that binds to and inhibits mTOR, resulting in inhibition of T lymphocyte activation and proliferation that occurs in response to antigenic and cytokine (IL-2, IL-4, and IL-15) stimulation and inhibition of antibody production
	Everolimus OSI-027	Binds to and inhibits both the raptor-mTOR complex 1 (TORC1) and the rictor-mTOR complex 2 (TORC2), resulting in tumor cell apoptosis and inhibition of tumor cell proliferation
Dual PI3K/mTOR	XL765/SAR245409 BEZ235 GDC-0980	Inhibits both PI3K kinase and mTOR kinase, which may result in tumor cell apoptosis and growth inhibition in susceptible tumor cell populations.
	GSK2126458	Binds to and inhibits PI3K in the PI3K/mTOR signaling pathway, which may trigger the translocation of cytosolic Bax to the mitochondrial outer membrane, increasing mitochondrial membrane permeability and inducing apoptotic cell death

	SF1126	Selectively binds to cell surface integrins and, upon cell entry, the agent is hydrolyzed to the active drug SF1101; Inhibits all isoforms PI3K, mTOR and DNA-PK, wich may inhibit tumor cell and tumor endothelial cell proliferation and survival
CDK4/6	LEE011 LY2835219 Palbociclib	Specifically inhibits CDK4 and 6, thereby inhibiting Rb protein phosphorylation, that prevents CDK-mediated G1-S phase transition, thereby arresting the cell cycle in the G1 phase, suppressing DNA synthesis and inhibiting cancer cell growth
Src	Dasatinib	Binds to and inhibits the growth-promoting activities of SRC-family protein-tyrosine kinases
Met	Tivantinib	Binds to the c-Met protein and disrupts c-Met signal transduction pathways, which may induce cell death in tumor cells overexpressing c-Met protein or expressing consitutively activated c-Met protein
IGF1R	Ganitumab	Binds to membrane-bound IGF-1R, preventing binding of the ligand IGF-1 and the subsequent triggering of the PI3K/Akt signaling pathway; inhibition of this survival signaling pathway may result in the inhibition of tumor cell proliferation and the induction of tumor cell apoptosis
HSP90	XL888	Specifically binds to Hsp90, inhibiting its chaperone function and promoting the proteasomal degradation of oncogenic signaling proteins involved in tumor cell proliferation and survival; inhibition of tumor cell proliferation may result
CTLA-4	Ipilimumab	enhances T-cell activation and blocks B7-1 and B7-2 T-cell co-stimulatory pathways
PD-1	Nivolumab	Binds to and blocks the activation of PD-1, an Ig superfamily transmembrane protein, by its ligands PD-L1 and PD-L2, resulting in the activation of T-cells and cell-mediated immune responses against tumor cells or pathogens
PD-L1	MDX-1105 Pembrolizumab MPDL3280A	Binds to PD-1, an inhibitory signaling receptor expressed on the surface of activated T cells, and blocks the binding to and activation of PD-1 by its ligands, which results in the activation of T-cell-mediated immune responses against tumor cells
Interleukin-2	Aldesleukin	Binds to and activates IL-2 receptor; activation of tyrosine kinase Jak3; and phosphorylation of tyrosine residues on IL-2R beta chain, resulting in an activated receptor complex. may induce T cell-mediated tumor regression in some tumor types

Table 1: Principal targeted therapies in completed or on-going clinical trials

Targeting BRAF

BRAF is the second kinase in the cascade of MAPK pathway: the identification that a high percentage of melanomas is driven by oncogenic BRAF has led to explore the most effective ways of inhibiting the constitutively active MAPK pathway. From these studies, BRAF inhibitors have been confirmed to represent the most promising agents for treating BRAF-mutant melanomas.

The first drug developed against BRAF was the BAY 43-9006 or sorafenib, which is however unspecific for mutated BRAF and suppresses activity of several different kinases (indeed, it is recognized as a multikinase inhibitor) [27]. This lack of target specificity produced a negative consequence on the outcome of the treatment of melanoma: in fact sorafenib was proven to be clinically ineffective as either single agent or in combination with chemotherapeutic drugs (i.e. carboplatin and paclitaxel) [28-30].

Vemurafenib (PLX4032), a second generation anti-BRAF compound that acts as potent and selective inhibitor of mutated BRAF kinase, has been demonstrated to be highly effective in melanoma patients carrying the V600EBRAF mutation [31]. Based on outstanding results shown from phase I and II studies, a randomised phase III study of vemurafenib compared to dacarbazine as standard treatment was launched and rapidly completed [32]. As hypothesized, patients treated with vemurafenib presented an overall survival at 6 months of 84% (95% CI: 78-89) as compared to patients treated with dacarbazine showing an overall survival of 64% (95% CI: 56-73) [32]. Additionally, patients from vemurafenib group presented a relative reduction of 63% in the risk of death and of 74% in the risk of either death or disease progression, as compared with those undergoing dacarbazine treatment [32]. Reproducing the same good clinical activity of vemurafenib, dabrafenib (previously known as GSK2118436) has been recognized as an additional potent and specific BRAF mutant inhibitor: it significantly improved progression-free survival as compared with dacarbazina [33]. Interestingly, dabrafenib seems to be equally active on different mutations at codons 600 of the BRAF gene (V600E/K/D/R) [33-35]. Overall, a clinical benefit has been reported up to an unprecedented 80% rate of BRAF-mutated patients treated with vemurafenib or dabrafenib; response to each of these oral agents occurs within few days or weeks [36].

In addition to the inhibitory activity in BRAF-mutant cells, which is revealed by the decreased level of phosphorylated ERK1/2 proteins and consequent growth arrest, vemurafenib and dabrafenib also can activate MAPK pathway in tumour cells with a wild-type BRAF through RAF-mediated induction of ERK1/2 phosphorylation [37,38]. It has been shown that wild-type RAF kinase activation induces RAF dimerization with paradoxical increase in MAPK signalling as result of increased dimer formation (CRAF-CRAF or BRAF-CRAF) that in turn activates MEK and, subsequently, ERK. This process is enhanced by the presence of an oncogenic RAS mutation [38-41]. The paradoxical activation of ERK might explain the formation of keratoacanthomas and squamous cell carcinomas among patients treated with BRAF inhibitors as well as the development of an acquired resistance to these drugs [38,41]. In fact, even though the response rates are high, the duration of response has been limited due to development of resistance: the median duration of response was 6 to 8 months.

Targeting MEK

Since reactivation of the downstream MEK-ERK pathway seems to represent the main mechanism of resistance to BRAF inhibitors, a promising strategy for overcoming such a limited persistence of the antiproliferative effects was to introduce new compounds blocking MEK1/2 proteins into the treatment options; indeed, several MEK inhibitors have been tested in clinical trials. While BRAF inhibitors only inhibit ERK signalling in cells with mutant BRAF, MEK inhibitors block ERK pathway in both tumour and normal cells. As single agents, these compounds (AS703026, AZD6244, E6201, GSK1120212, GDC0973, MEK162) have shown a markedly high activity in patients carrying tumours with constitutive activation of the RAS/BRAF/MEK/ERK signalling cascade. Detection of RAS mutations in primary tumours seems to represent the strongest marker for selecting patients with the highest chance to respond to MEK inhibitors; AS703026 and AZD6244 have activity in KRAS mutant colon cancer cell lines/xenografts in combination with cetuximab [42,43], whereas GSK1120212 (also known as trametinib) has been found to be effective in NRAS-mutated melanoma [44]. In melanoma patients carrying BRAF mutations, the response to MEK inhibitors seems to be partially dependent on exposition to prior therapy with BRAF inhibitors; for GSK1120212, a significant clinical activity was observed in BRAF-inhibitor-naïve patients only [44,45]. Similarly, the response seems to hinge on status of the PI3K-AKT pathway: for selumetinib (AZD6244) and E6021 a significantly low responsiveness to MEK inhibitors was found in BRAF mutant melanomas expressing high levels of phosphorylated AKT [46] or presenting PTEN inactivation with subsequent stimulation of downstream PI3K signalling [47], respectively. In other words, coexistence of an unaffected PI3K-AKT status may contribute to increase sensitivity to MEK inhibitors in melanomas whose MAPK pathway is activated through oncogenic mutations in BRAF gene. Finally, the MEK inhibition has been demonstrated to abrogate the CRAF-dependent activation of ERK in wild-type BRAF cells, contributing to reduce the chances of cutaneous adverse events [48]. Current clinical investigations have shown great promise with the combination of targeted therapies as a new effective strategy of melanoma treatment. A combined treatment with MEK and BRAF inhibitors in BRAF mutated metastatic patients showed a significant improvement of the progression-free survival rates [49], providing further support to the hypothesis that this could be the way for a better management of such melanoma cases. Actually, a number of clinical trials of trametinib in combination with other targeted drugs, whose activity is somehow interfering with the MAPK-driven tumour growth, are underway and expected to show great promise. As an example, it has been recently demonstrated that MEK inhibitors may enhance the ability of histone deacetylase (HDAC) inhibitors to induce apoptosis in tumour cells with constitutive activation of the BRAF-MEK-ERK signalling cascade both in vitro and in vivo [50].

Tables 2 [9,10,49,51-54] and 3 [9,10,49,51,55] report the main clinical trials with targeted agents for treatment of advanced melanoma.

Mechanisms of Resistance to MAPK-Targeted Therapy.

Although the antitumor effects of target therapy are striking, intrinsic and acquired resistance limits the therapeutic benefit of this approach [6,56]. On this regard, is it to be underlined that vast majority of data about such an issue is related to the resistance to BRAF inhibitors, since vemurafenib and dabrafenib have been the most extensively studied, both preclinically and clinically.

Reference	Trial	No. of pts	Target	Molecular alteration	Agent(s)	Clinical Benefits	Adverse events
Chapman 2011	Phase III	675	BRAF	$BRAF^{V600E}$ mut	Vemurafenib	ORR: about 50%; PFS: 5.3 months; OS: 84% at 6 months	arthralgia, fatigue, cutaneous events, squamous cell carcinoma (SCC), keratoacanthoma (KA), or both
Hauschild 2012	Phase III	250	BRAF	$BRAF^{V600}$ mut	Dabrafenib	ORR: about 60%; PFS: 5.1 months; OS: 42% at 5 months	arthralgia, pyrexia, fatigue, headache, hyperkeratosis, papillomas, palmar-plantar erythrodysesthesia, SCC, KA, basal cell carcinoma, mycosis fungoides
Robert 2012	Phase III	322	MEK	$BRAF^{V600}$ mut	Trametinib	ORR: about 22%, PFS: 4.8 months; OS: 81% at 6 months	rash, diarrhea, nausea, vomiting, fatigue, peripheral edema, alopecia, hypertension, constipation, central serous retinopathy and retinal-vein occlusion
Flaherty 2012	Phase I/II	247/162	BRAF+ MEK	$BRAF^{V600E/K}$ mut	Dabrafenib+ Trametinib	ORR: about 76%, PFS: 9.4 months; OS: 41% at 12 months	pyrexia, chills, fatigue, rash, nausea, vomiting, diarrhea, abdominal pain, peripheral edema, cough, headache, arthralgia, night sweats, decreased appetite, constipation, and myalgia
Ascierto 2013	Phase II	71	MEK	$BRAF^{V600}$ mut	MEK162	PRR: about 20%, OR+SD: 52%	rash, diarrhea, acneiform dermatitis, creatine phosphokinase (CK) elevation, fatigue, peripheral edema, central serous retinopathy-like retinal events.
				NRAS mut		PRR: about 20%, OR+SD: 63%	
Catalanotti 2013	Phase II	15	MEK	$BRAF^{V600e/k}$mut	Selumetinib	ORR: about 11%, PFS and OS: data pending	rash, fatigue, elevated liver function tests, lymphopenia, hypoalbuminemia, dyspnea, cardiac function

Carvajal 2013	Phase II	48	MEK	GNAQ mut	Selumetinib	ORR: about 15%, PFS and OS: data pending	CPK elevation, LFT elevation, rash, lymphopenia, edema

Table 2: Targeted therapy single agents and combinatorial testing in melanoma

Intrinsic resistance

Approximately 20% of patients with BRAF mutated melanoma tumours are not responsive to the treatment at all (vemurafenib or drabafenib) due to intrinsic resistance [13]. Because melanoma exhibits a wide spectrum of tumour-associated genomic lesions and a high degree of inter- and intratumoral heterogeneity, the mechanisms of intrinsic resistance to RAF inhibitors are likely to be diverse:

- Gene amplification and/or overexpression of Cyclin D1, which contrasts the activity of the cyclin-dependent kinase inhibitor p16CDKN2A and stimulates the CyclinD1-RB pathway [57] (Table 3);

Reference	Targeted therapy	Number of patients (ratio)	Patient population	Response rate (95% CI)	Median PFS	Median OS	6-month OS rate	12-month OS rate
Chapman 2011	Vemurafenib	675 (1:1)	Previously untreated	48% (42–55)	6.9 months	13.6 months	84%	56%
	Dacarbazine			5% (3–9)	1.6 months	9.7 months	64%	44%
Hauschild 2012	Dabrafenib	250 (3:1)	Previously untreated (except HD IL-2)	50% (42–57)	5.1 months	18.2 months	NR	NR
	Dacarbazine			6% (2–16)	2.7 months	15.6 months	NR	NR
Robert 2012	Trametinib	322 (2:1)	One previous treatment allowed, except BRAF or MEK inhibitors or ipilimumab	22% (17–22)	4.8 months	NR	81%	NR
	Dacarbazine or paclitaxel			8% (4–15)	1.5 months	NR	67%	NR
Flaherty 2012	Dabrafenib+trametinib (150 mg/1mg)	247 (1:1:1)	One previous treatment allowed, except BRAF inhibitor	50% (36-64)	9,2 months	NR	NR	26%
	Dabrafenib+trametinib (150 mg/2mg)			76% (62-86)	9.4 months	NR	NR	41%
	Dabrafenib			54% (40-67)	5.8 months	NR	NR	9%
Robert 2012	Selumetinib+Dacarbazina	91 (1:1)	Previously untreated	40% (18-45)	5.6 months	13.9 months	40%	NR
	Dacarbazina			26% (12-46)	3 months	10.3 months	22%	NR

Table 3: Summary of phaseII/III randomized trials for melanoma

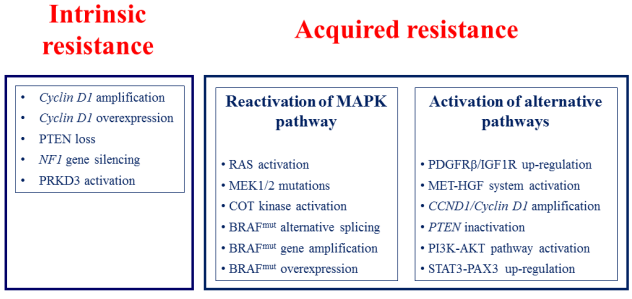

Figure 1: Main known mechanisms (intrinsic and acquired) by which BRAF mutant cells develops drug resistance against RAF and MEK inhibitors.

- Less of PTEN tumour suppressor protein and consequent increased basal level of AKT signalling [58];
- Silencing of the NF1 gene, which either promotes RAS activation either impairs the mechanisms regulating the senescence process controlling the cell proliferation [59];
- Increased activity of protein kinase D3 (PRKD3), with activation of the PI3K-AKT signalling in presence of a specific inhibition of the oncogenic BRAF [60].

Figure 1 summarizes the different resistance mechanisms which are preexistent (giving an intrinsic refractoriness) or activated following the drug administration (favoring the block escape in a MAPK-dependent or MAPK-independent manner) to the treatment with inhibitors of the RAS-RAF-MEK-ERK pathway.

To better understand the reasons why all these apparently different molecular alterations are implicated in conferring resistance to BRAF or MEK inhibitors in melanoma cells, it is necessary to keep in mind the relationship between RAF/MEK/ERK activation and melanomagenesis. As is common knowledge, oncogenic BRAF mutant strongly stimulates cell cycle progression by activation of downstream MEK/ERK pathway. However, the BRAF-driven melanocytic proliferation needs the coexistence of alterations in additional cell-cycle factors (such as p53 deficiency, genetic/epigenetic inactivation of p16CDKN2A gene, increased levels of active AKT) in order to promote melanoma growth and progression [61]. In a subset of melanomas, such additional pathogenetic alterations acquire a prevalent role and tumour cell proliferation becomes independent or less dependent on activation of BRAF/MEK/ERK pathway.

Independently from the functional status BRAF/MEK/ERK pathway, overexpression of Cyclin D1 may drive cell cycle entry and uncontrolled growth: increased Cyclin D1 protein expression determines a marked increase in activating bind to the CDK4/6 kinases and in phosphorylation of the RB protein. In support of this overexpression experiments showed that introduction of Cyclin D1 into previously drug sensitive cell lines did facilitate cell cycle entry and proliferation even when BRAF was inhibited [57].

As mentioned above, the loss of PTEN function results in accumulation of PIP3 mimicking the effect of PI3K activation and triggering the activation of its downstream effectors like AKT. Hyper-activated AKT has been shown to promote cell proliferation, possibly through down-regulation of the cyclin-dependent kinase inhibitor p27 and the up-regulation of Cyclins E and D1 [62,63]. Differential mechanisms of AKT activation demand an upstream PI3K activation, since activating mutations of AKT are nearly absent in melanoma (only rare mutations in AKT1 and AKT3 genes have been indeed reported in a limited number of melanomas and melanoma cell lines [64-66]). On this regard, the PI3K signalling seems to be directly increased by the occurrence of activating mutations in its kinase domain [67].

AKT regulates the apoptotic response to a variety of stimuli via its ability to interact with a number of key players in the apoptotic process, its intracellular accumulation does result in the suppression of apoptosis and induction of cell survival [64]. AKT can directly phosphorylate BAD (Bcl-2 antagonist of cell death) and MDM2; in turn, BAD inactivation affects the interaction of this protein with with anti-apoptotic members of the Bcl-2 family of proteins (Bcl-2, Bcl-XL) [68-69] and MDM2 leads to increases p53 degradation [70,71]. In addition, the increase in AKT signalling suppresses the expression of BIM (pro-apoptotic member of Bcl-2 protein family), with inhibition of its pro-apoptotic activity [72]: the expression levels of BIM protein is indeed regulated by silencing of PTEN and subsequent activation of the PI3K-AKT pathway in conjunction with the activation of BRAF/MEK/ERK pathway [73]. The presence of PTEN inactivation may therefore interfere with the BRAF inhibition by reducing the levels of BIM protein and, thus, the extent of apoptotic induction; as a confirmation of this, a simultaneous treatment with BRAF and PI3K inhibitors has been reported to enhance BIM expression and increase the level of induced apoptosis [58].

Hence, the occurrence of a p53 deficiency or, more in general, a status of apoptosis escape, with an unbalanced ratio between pro- and anti-apoptotic effectors-all events found to cooperate with BRAF mutations in driving the melanoma progression [74-75]-may induce a MAPK-independent tumour growth [76]. Inactivation of AKT by targeting PI3K has been also demonstrated to effectively inhibit cell proliferation [58,77]. The combination of a BRAF or MEK inhibitor with a PI3K/mTOR inhibitor was found to enhance cell growth inhibition through achievement of ERK hypophosphorylation, reduced Cyclin D1 levels and increased p27 levels, overcoming the resistance encountered by the use of a single anti-BRAF or anti-MEK agent [44,78]. Amplification of Cyclin D1, allelic deletions down-regulating p16CDKN2A, and alterations inactivating PTEN have been all associated with a poorer progression-free survival following treatment with dabrafenib in patients with BRAF-mutant metastatic melanoma [79].

The protein encoded by the NF1 gene, neurofibromin, is a known tumour suppressor gene and negative regulator of RAS protein. Therefore, loss of NF1 mediates resistance to RAF and MEK inhibitors trough sustained MAPK pathway activation [59]. Recent studies have shown how NF1 ablation decreases the sensitivity to BRAF inhibitors in BRAF mutant melanoma cells that are intrinsically resistant to BRAF inactivation as well as in melanomas developing resistance to vemurafenib [59,80].

Finally, activation of PRKD3 (protein kinase D3) contribute to resistance to such target therapies by direct stimulating the PI3K-AKT pathway. Inhibition of this gene has been reported to enhance cell growth arrest by BRAF and MEK inhibitors and enforce cell sensitivity to these agents [60]. The NF1 loss and the PRKD3 activation can be considered as key mediators of both acquired and intrinsic BRAF inhibitor resistance (increased activity of PRKD3 seems to however confer resistance to RAF265 rather than approved BRAF inhibitors [60]).

Acquired resistance

Although very encouraging, the clinical responses to BRAF inhibitors are relatively short-lived and resistance to treatment develops in 6 to 8 months from the initiation of therapy, with treatment failure and tumour progression occurring in nearly every case. In contrast with several studies that shown how acquired drug resistance was associated with the acquisition of secondary mutations in kinase being targeted that prevented the binding of drug (for example T790M in the EGR receptor [81] and T315I in Bcr-ABL [82]), secondary BRAF mutations were not the mechanism of resistance in melanomas patients [40]. The emerging data instead suggest that a diverse array of BRAF inhibitor acquired resistance mechanisms exists and they are highly heterogeneous [83].

At a glance, two different pathogenetic scenarios of acquired resistance may be depicted (Figure 1).

The first scenario include mechanisms underlying reactivation of the RAS/RAS/MEK/ERK pathway trough induced alterations in components of this signalling cascade: activation of RAS signalling [84], activating mutations in MAP2K1 (encoding MEK1 protein) or MAP2K2 (encoding MEK2 protein) genes [85,86], activation of MAPK pathway agonists such as COT kinase [87], occurrence of alternative splicing of the BRAF mutant mRNA [88], BRAF-mutated gene amplification [89]. In this case, the cell proliferation/tumour growth is still depending on RAS/BRAF/MEK/ERK cascade activity and BRAF inhibition is overcome with alternative changes within this same pathway (real failure of BRAF inhibitors).

The second scenario is represented by reactivation of the suppressed ERK signalling through induced alterations in components of cell proliferation-controlling pathways different from the BRAF/MEK/ERK cascade: up-regulation of the receptor tyrosine kinase (RTK) effectors-such as the platelet-derived growth factor receptor β (PDGFR β) [90], activation of the MET-HGF system [91], amplification of the CCND1/Cyclin D1 gene or lack of PTEN function with subsequent activation of the PI3K-AKT pathway [62], enhancement of the IGF-1R/PI3K signalling [92], up-regulation of the signal transducer and activator of transcription 3 (STAT 3)-paired box homeotic gene 3 (*PAX 3*)-signalling pathway [93-95]. In this case, BRAF inhibition is still effective, but the tumour is not anymore dependent upon RAF/MEK/ERK signalling for growth and survival (paradoxical failure of BRAF inhibitors).

As largely known, in melanoma with mutated BRAF, activation of the downstream MEK/ERK pathway is independent on the RAS-

ligand activity and BRAF mutant transmits continuous proliferation signals acting as a RAF-inhibitor-sensitive monomer. Vemurafenib and dabrafenib potently inhibit such BRAF mutant monomers, causing markedly decreased levels of ERK phosphorylation [94]. As a consequence, the ERK-dependent feedback is progressively turned off, RAS-driven signal transduction is restored with increasing levels of active RAS-GTP, and RAF-inhibitor-resistant RAF dimers are generated. The RAF homo- or heterodimers (CRAF-CRAF, BRAF mutant-CRAF) are able to reactivate the MEK/ERK pathway with a consequent increased activity of the ERK 1/2 proteins [90-96]. In preclinical models, increased activity was firstly identified in drug-resistant clones derived from cell line undergoing BRAF-inhibition [97]. Occurrence of CRAF mutations has been to also contribute in reactivating the MEK-ERK axis - again, in a dimerization-dependent manner-following exposure to RAF inhibitors [98]. Enhanced RAS-dependent RAF dimerization has also been involved into the pathogenesis of squamous cell carcinomas, as a side effect in subsets of patients treated with RAF inhibitors [99,100]. These agents have been demonstrated to indeed activate MAPK pathway by inducing RAF dimerization in cells lacking BRAF mutations [38,40,88,102], leading to increased keratinocyte proliferation. Enhanced RAF dimerization is also promoted by alteration such as NRAS mutations: a genetic analysis of biopsies from patients resistant to vemurafenib revealed the presence of an activating NRAS mutation (Q 61) that was lacking in the original tumours. This switch in mutational status was accompanied by the reactivation of MAPK pathway after treatment with vemurafenib [40]. Mutations in any of the three isoforms of RAS (with preponderance of those occurring in HRAS gene) may also contribute to the development of squamous cell carcinomas as adverse event during the treatment with BRAF inhibitors [5,40].

Another mechanism that drives formation of RAF dimers, with consequent RAF inhibitor resistance, consists in upstream activation of receptor tyrosine kinase (RTK) MET via hepatocyte growth factor (HGF), which is its main ligand [91,103-104]. Several studies has shown how HGF, overexpressed by stromal cells of the tumour microenvironment, stimulates MET receptor promoting transduction of the signal to the downstream PI3K effector with subsequent enhancement of AKT activity [104,105]. The HGF-MET axis plays a critical role in both intrinsic and acquired resistance to BRAF inhibition; the addiction of either an inhibitor of HGF or MET simultaneously to BRAF inhibitor re-establishes sensitivity to BRAF inhibition [91]. To investigate if additional pathways were stimulated in response to chronic BRAF inhibition, the activation of several tyrosine kinase receptors (RTKs) are being examined; among them, insulin like growth factor receptor-1 (IGF-1R) has been identified as being constitutively activated in resistant cells [92]. IGF-1R can activate both the MAPK and PI3K pathways: IGF-1R signalling cooperate with MAPK pathway in regulating progression from benign nevi to malignant melanoma through sustainment of cell survival and dissemination and increase of IGF-1R expression reflects an enhanced activity of PI3K/AKT [92,106]. All these clues suggest the possible existence of a negative crosstalk between the two pathways during chronic BRAF inhibition. Crosstalk between MAPK and PI3K has been reported in several cancer systems, but not much is known in melanoma [107,108]. Interruption of IGF-1R signalling has been shown to inhibit tumour growth and block metastasis formation in a wide variety of tumour models and dual inhibition of IGF-1R and MEK inhibitors has been demonstrated to induce growth arrest in BRAF inhibitor-resistant cells [92]. Acquired resistance to BRAF and MEK inhibitors resistance seems to be associated with the up-regulated expression of other RTKs such as PDGFR β–receptor (platelet derived growth factor receptor): its increased expression, in BRAF-inhibitor resistant cellular models, was demonstrated to be responsible for improving cell survival and invasiveness in a manner independent on the activation of the MAPK pathway [40]. In presence of BRAF or MEK inhibitors, IFG-1R and PDGFRβ signalling has been shown to overexpress the STAT3 (transcriptional activation factor) with consequent activation of STAT3 pathway through stimulation of the Src/FAK transducers [94,109-111]. STAT 3 acts as a direct transactivator of the PAX3 promoter implicated in activating expression of the receptor tyrosine kinase MET in melanoma [112]. The importance of the STAT3-PAX3 signalling axis has been highlighted through knockdown experiments; indeed, knocking down either STAT3 or PAX3 in vemurafenib resistant BRAF mutated melanoma cells reduced cancer proliferation [93]. Conversely, upregulation of STAT 3 allows cells to become independent on the activity of the BRAF-MEK pathway and contribute to resistance to BRAF and MEK inhibitors [93-94,113]. Nearly all results about the role of the RTK effectors in resistance to such targeted treatments have been obtained in studies on melanoma cell lines; therefore, significant data from analysis of clinical samples are not yet available.

Through a functional genomics approach, expression of COT kinase was also identified as a putative mechanism of RAF inhibitor resistance: a number of melanoma cells lines and tissues showed a genomic amplification of COT associated with intrinsic BRAF and MEK inhibitor resistance [87]. The overexpression of the COT kinase, which is encoded by the MAP3K8 gene, is induced by the treatment with BRAF or MEK inhibitors acting as an agonist of the MAPK pathway and leading to resistance to BRAF-MEK inhibition [87]. The identification of COT is an example of an inhibitory bypass mechanism that results in reactivation of ERK signalling in a RAF-independent manner. However, the relevance of increased COT expression in the resistant phenotype was mainly evidenced in experimental sets but poorly confirmed in patients failing BRAF and MEK inhibitor treatment. Downstream mutations in MAP2K1 (encoding MEK1 protein) and MAP2K2 (encoding MEK 2 protein) genes have also been reported as resistance drivers to BRAF or MEK inhibitors [115]. Specially the MEK1 P124L and Q56P specific mutations have been shown to modify the allosteric pocket of MEK1, making MEK1 protein either independent on stimulation by upstream BRAF either insensitive to MEK inhibitors [85]. Further study have shown how other MEK1 mutants (e.g. P142S and I111S) respond to BRAF inhibitor treatment, suggesting that not all MEK1 mutations make BRAF–mutant melanomas resistant to BRAF inhibitors [115].

While secondary mutations in BRAF have not been identified as a cause of BRAF or MEK inhibitor resistance (see above), several studies identified selective amplification of the mutant BRAF allele as the mechanism underlying acquired resistance [116-118]. Gene mutations and copy number gains may occur independently of each other, since are determined from different pathogenetic mechanism: alterations affecting the molecular machinery that monitors the proper progression of the cell cycle seem to be responsible for the presence of gross genomic anomalies during the malignant progression (indeed, copy number gains are often the consequence of random genomic instability), whereas mutations usually occur in diploid karyotypes with few structural abnormalities during the initial phases of evolution of malignancies [119]. However, in some cases, gene amplifications tend to occur in the same cancers presenting oncogenic mutations, as reported for EGFR in NSCLC or BRAF in colorectal carcinoma [120-121]. Recent elegant study has shown how melanoma cells

chronically exposed to trametinib acquired concurrent MEK2-Q60P mutation and BRAF-V600E amplification, which conferred resistance to MEK and BRAF inhibitors [122].

A peculiar, qualitative mechanism of resistance is represented by the intracellular accumulation of a splice variant of the mutated BRAF mRNA. A subset of melanoma cells resistant to BRAF inhibitors express a truncated form of BRAFV600E, p61BRAFV600E, that lacks the RAS-binding domain but retain the kinase domain [88]. These BRAF splice variants dimerize in a RAS-independent manner, consistent with the model that only BRAF V600E monomers are sensitive to inhibition. The final effect of such an alteration is a trans-activation of the MEK-ERK pathways, with ERK signalling being resistant to the RAF inhibitors [41,88]. Moreover, the vemurafenib-resistant melanomas presenting an enhanced transcription and translation of the mutated BRAF kinase may develop a drug dependency for their continued proliferation, such that cessation of BRAF inhibitor administration may lead to regression of non-lethal drug-resistant tumours [123]. This evidence has suggested that a discontinued treatment with these agents may somehow prevent the emergence of lethal drug-resistant cell clones [123]. Although BRAF splice variants were not detected in vemurafenib-naïve patients with cancer, it is possible that they are expressed in a small subpopulation of cells within the pre-treatment tumour and that exposure to BRAF inhibitors provides a selective pressure for the propagation of the BRAF splice variant–expressing tumor population. More in general, since major genetic alterations (i.e. mutations in BRAF and NRAS) are maintained during melanoma progression [124,125], one could speculate that resistance to targeted therapies are likely due to the presence of resistant sub-clones within the primary tumors which may be induced to proliferate and expand themselves after the initiation of inhibitory therapy (Figure 2). On this regard, much debate however exists in regard to the "selection" or "acquisition" of molecular alterations conferring resistance to targeted therapies in different types of cancers. The fact that in rare instances the resistance alterations have been identified in biopsy specimens of treatment-naive patients using standard screening techniques could be indeed imputed to either the true initial absence of them or the poor sensitivity of the current analytical methods in identifying the very limited fraction of tumor cells with such under-represented alterations. Further supporting the hypothesis about the prevalence of the "selection" model of resistance alterations, a growing number of studies are suggesting that the inherent phenotypic and genetic heterogeneity of cancer cell populations in primary tumours represent a critical determinant of drug resistance [126,127].

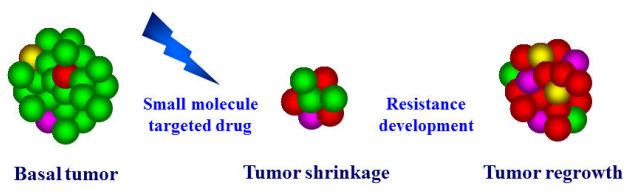

Figure 2: Model of tumor drug resistance. Due to molecular heterogeneity of melanoma, after exposure to target inhibitors, the drug-resistant cells survive and proliferate, giving rise to a tumor made up of the progeny of the drug-resistant cells.

Future Perspectives

Although the resistance mechanisms identified so far are multiple, it is evident that a crucial role in determining such a phenomenon is played by the increased activity of ERK or AKT signalling. As seen above it is possible that reactivation of ERK or AKT survival pathways-as a result of aberrations in regulators of BRAF activity-may replace the tumor's initial oncogene addition. This may complicate the outcome of the targeted therapy since the BRAF-mutant melanomas may no longer be responsive to treatment with a single BRAF inhibitor. In support of this, activation of the ERK1/2 proteins and, therefore, of the ERK-dependent nuclear transcription has been largely reported to significantly drive either the development of an acquired drug resistance or the occurrence of most of the side effects in melanoma patients. In preclinical models, a selective, ATP-competitive inhibitor of ERK1/2 kinases has been described to resume growth suppression in melanoma cells whose resistance was determined by ERK reactivation [128].

The activation of multiple signaling pathways by many genes illustrates the need for the targeting of more than one signaling pathway. In most cases, the addition of a compound directed against one of these latter activated effectors to the treatment with a targeted agent may contribute to overcome resistance to single inhibitors as demonstrated by discovery that new RAF inhibitor are able to both inhibit ERK activity and protect ERK1/2 kinases from NRAS-driven reactivation in vemurafenib-resistant cells [129]. The addition of a MEK inhibitor to the RAF inhibitor seem to increase the magnitude and/or durability of response, as shown in several studies [37,47,48,130,131].

As inferred by studies on RAF inhibitor resistance, another rational strategy could be represented by a combination of inhibitors co-targeting components of the PI3K-AKT pathway; pre-clinical data seem to suggest that such a treatment may become a winning therapeutic strategy to exert an effective antitumor outcome in melanoma patients. In this sense, combined treatment based on inhibition of BRAF and silencing of AKT3 was found to significantly increase suppression of tumour growth as compared to the result obtained by single agent administration [78,132,133]. In in vivo melanoma models, the synergistic use of MEK and PI3K inhibitors is more potent and more effective in overcoming antitumor resistance [134,135], as also confirmed by a study where the clinical relevance of the dual-targeting strategy involving MEK and PI3K inhibitors was evaluated in patients with advanced cancer [136]. Similarly, the combination of MEK inhibitors with agents inhibiting mTOR, the downstream effector of the PI3K-AKT pathway, has been reported to exert an effective antitumor response inhibiting tumor growth, inducing cell death, and abrogating invasiveness of melanoma cells. [44,137,138]. Taken together these results indicate that combinations of inhibitors as PI3K (upstream) or mTOR (downstream) suppressed AKT activity and enhance the antitumor effectiveness of the MAPK-targeted therapies.

The existence of so many potential resistance mechanisms requires complex patient-specific approaches to either more accurately classify from the molecular point of view all cases to be addressed to targeted therapies either develop new combinational treatment with multiple gene inhibitors that may help in overcoming toxicities and resistance.

Conflict of Interest

All authors declare the absence of any Conflict of Interest.

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